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Chapter

Prevalence, Antimicrobial Resistance and Pathogenicity of Non-O1 *Vibrio cholerae* in Suburban and Rural Groundwater Supplies of Marrakesh Area (Morocco)

Hafsa Lamrani Alaoui, Khalid Oufdou and Nour-Eddine Mezrioui

Abstract

This synthesis of research work considers the dynamic, antibiotic resistance, hemolytic, and hemagglutination activities of non-O1 *Vibrio cholerae* in comparison with those of fecal coliforms, fecal streptococci, and *Pseudomonas aeruginosa* isolated from suburban and rural groundwater supplies in a Marrakesh area (Morocco). In addition, it assesses the influence of some chemical factors on the distribution of all these bacterial groups. The obtained results showed that the prospected well waters contain them at varying abundance degrees while undergoing generally spatial and temporal fluctuations. The total occurrence of these bacteria during the period of study was 94%. Detectable non-O1 *V. cholerae* was present in 81% of the samples and the mean abundances ranged from 0 to 11100 MPN/100 ml. According to WHO standards for drinking water, they were heavily contaminated and could have significant health risks for the local population consuming them. Non-O1 *V. cholerae* and the other studied bacteria are virulent since most of them were found to be adhesive, producers of hemolysins and multi-resistant to antibiotics. Pollution activities around the wells lead to an increase of virulence and antimicrobial resistance in groundwater. This shows the role of these bacteria in several cases of gastro-enteric and systemic pathologies noted in Marrakech local population.

Keywords: non-O1 *Vibrio cholerae*, antibiotic resistance, groundwater, hemagglutination, hemolysin, chemical factors

1. Introduction

Currently, pollution, water scarcity and seasonal droughts are emerging as major development challenges for many developing countries. According to data for the world's most water-stressed countries [1], Morocco is among the most vulnerable and will become a water-stressed country by 2040 [2, 3]. Thus, the preservation and management of its aquifers against various forms of pollution is a major national and

regional concern. In Marrakesh area, groundwater supplies are a valuable resource for wide suburban and rural populations. However, their consumers' growth and anthropogenic activities heavily influence these wells water. In fact, due to the lack of sewerage networks and the absence of household waste collection, these people are directly discharging wastewater and solid waste onto the ground. Then, the chemical and microbial quality of well water under such environments is seriously threatened. Also, these communities rely only on these untreated wells' water as a source of human and animal drinking, domestic activities, and cultural irrigation. The use of these well waters without any previous treatment involves serious health problems due to the potential presence of pollutants and pathogenic bacteria. Water pollution has a direct impact on human health so that about 884 million people are living without access to clean drinking water in 2019 [4, 5]. According to WHO, about 1.8 million people die every year because of cholera and diarrhea, and 3900 children die every day as a result of contaminated water consumption and sanitation conditions inadequacy [6, 7]. Indeed, emergences of some aquatic diseases and sporadic outbreaks of acute diarrhea were reported on several occasions in Marrakesh area especially in the hot period [8, 9]. Even so, etiological information related to such outbreaks in this region is very limited. The occurrence of antibiotic-resistant bacteria in these wells' water could worsen more than in this previous situation. Many research studies have noted the important public health implications of the presence of antibiotic-resistant bacteria and especially multiple antibiotic-resistant bacteria, in suburban and rural groundwater [10, 11]. The horizontal gene transfer and clonal spread of resistant bacteria can mediate the transfer of not only antibiotic resistance genes but also virulence factors [12, 13].

There is a growing trend toward infection due to *Vibrio* spp., their capacity to persist in the aquatic environment and their association with abiotic and biotic factors [14, 15]. Non-O1 *Vibrio cholerae* is ubiquitously distributed in diverse aquatic environments where water acts as a reservoir and source of its transmission [16]. They are an essential and potentially life-threatening cause of infections that are primarily related to the consumption of feces-contaminated water and person-to-person transmission [17–19]. This pathogen leads to self-limiting gastroenteritis, septicemia, bacteremia, meningoencephalitis, oral infection, wound or ear infections, and non-epidemic diarrhea with a fatal outcome in immunocompromised hosts with predisposing medical conditions [19–23]. Different virulence factors have been suggested to be involved in these diseases such as a heat-stable toxin, hemolysin and other cell-associated hemagglutinins [24–26]. Few studies have been conducted in Morocco on the occurrence of antibiotic resistance and virulence factors of non-O1 *V. cholerae* in groundwater supplies in Morocco and particularly in the Marrakesh area. Accordingly, this study presents a synthesis of our research works on the dynamics, occurrence of antibiotic resistance, and potential virulence of non-O1 *V. cholerae* in supplying well water in comparison with other bacteria. The incidence of hemolytic and hemagglutination activities and the importance of some chemical parameters on the distribution of non-O1 *V. cholerae*, *Pseudomonas aeruginosa*, fecal coliforms (FC), and fecal streptococci (FS) is discussed.

2. Dynamics, pathogenicity of non-O1 *V. cholerae* in suburban and rural groundwater supplies in Marrakesh area (Morocco)

2.1 Dynamics and ecology of non-O1 *V. cholerae*

Diarrheic diseases caused by contaminated water continue to be a serious problem in developing countries and a lesser, but chronic problem in developed

countries [4, 5]. More than half of the reported waterborne disease outbreaks have been linked to contaminated groundwater [27]. The importance of the ecology, distribution, and pathogenicity of non-O1 *V. cholerae* in water ecosystems is underestimated in the Mediterranean region [28] and particularly in Morocco. In preliminary research, the bacteriological and physicochemical quality was studied in sixteen well waters [29]. The prospected wells were located in two regions (Tensift and Jbilet aquifers) with environments representing different vulnerability to contamination. Sampling stations are situated at the north of Marrakesh city. The hydrology of these regions was relatively similar but they were geologically different. In the Jbilet region where the 10 studied wells (W1, W2, W3, W4, W5, W6, W7, W8, W9, and W10) are situated, the geological formations are almost all represented by Schist [30]. They are altered on the surface and give a stony soil with a few centimeters of clay soil. However, runoff waters seep partly into cracked and altered areas and fault zones [30, 31]. In the Tensift region where the other 6 monitored wells are sited, there is a predominance of superficial limestone formations and very permeable alluvial deposits [32]. In these two areas, the groundwater contained in these permeable formations was relatively slightly deep (6–30 m). To study the dynamic of non-O1 *V. cholerae*, we have targeted essentially six wells depending on their level of fecal contamination, proximity to pollution sources. The other reasons were their relative importance for local populations, and given that the research methodology of this pathogen is arduous and pricey. We have also selected wells that are highly susceptible to degradation by anthropogenic activities. W11 was the control well as it's not damaged by any pollutant source categories. Taking into account all these factors and risks, we opt for the following wells to identify non-O1 *V. cholerae*: W2, W3, W5, W9, W11, and W14. Spatial and temporal distributions of non-O1 *V. cholerae*, *P. aeruginosa*, and fecal indicators abundances were conducted over a year. Sampling was done each month from April 2004 to April 2005.

For non-O1 *V. cholerae* enumeration, the three tubes MPN technique was accomplished to search this bacterium according to the methodology described by Mezrioui et al. [33], Mezrioui and Oufdou [12] and Lamrani Alaoui et al. [34]. Concentration by the membrane filtration technique (Millipore size, pore: 0.45 μm) of volumes of 100, 10, and 1 ml or their dilutions was the perfect way to inoculate non-O1 *V. cholerae* into three tubes using the MPN technique. It consists of three steps: alkaline peptone water enrichment step (1% peptone, 1% NaCl, pH 8.6) for 18 hours of incubation at 37 °C. Seeding stage on thiosulfate-citrate-bile-sucrose selective media (TCBS) with incubation at 37 °C for 24 h. Identification of colonies presumed to be non-O1 *V. cholerae* through screening tests cited previously.

The standard bacterial indicators of fecal pollution in waters are FC. They are the most used to know the bacterial load of groundwater and testify the potential presence of enteric pathogens in water. But, to differentiate the fecal contamination of human and animal origin, it is proposed to use FS [35–37]. *P. aeruginosa* is an environmental microorganism that has become a major cause of opportunistic infections. It is regarded as complicated and can be life-threatening, especially respiratory tract infection in patients with cystic fibrosis and corneal infection or septicemias and diarrhea [38]. The counts of FC, FS, and *P. aeruginosa* were carried out on filtered volumes by 100, 10, and 1 ml of water samples by the membrane filtration technique (Φ = 0.45 μm). The appropriate selective media to each bacterial group was used like the following: lactose 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) agar with tergitol 7 (Biokar Diagnostics) for FC, Slanetz agar with TTC (Pasteur Diagnostics) for FS, and Cetrimid agar medium (Merck Diagnostics) for *P. aeruginosa*. For FC, the yellow colonies were enumerated after incubation at 44.5 °C for 24 h. For FS, the pink colonies were enumerated after incubation at 37 °C for 24 h. For *P. aeruginosa*, the fluorescent colonies were enumerated after

incubation at 42 °C for 24 h to 48 h. The densities of these bacteria were expressed by the indirect count of colonies forming units (cfu). For the physicochemical analyses, water temperature, pH, salinity, conductivity, and dissolved oxygen were measured *in situ* by the multiline engine parameters P4 SET. The other measured parameters (nitrate, nitrite, ammonium, organic matter, sodium, chloride, calcium, potassium, and sulphates) were determined at the laboratory according to the methods described by Lamrani Alaoui et al. [29].

The dynamics of non-O1 *V. cholerae*, *P. aeruginosa*, FC, and FS were statistically analyzed using SPSS 10.0 for windows. The two factor analysis of variance (ANOVA) was used for seasonal variation and the difference between the stations' comparison of bacterial abundances. Bacterial densities were transformed into a log10 unit to realize data analysis. Significant differences between each pair of average were established when $p \leq 0.05$. While being based on the Spearman correlation test, the nature of relationships between bacterial abundances and physicochemical factors was performed. Densities variation of non-O1 *V. cholerae*, *P. aeruginosa*, FC, and FS underwent high spatial and temporal fluctuations. The degree of pollution in these wells was different. Detectable non-O1 *V. cholerae* was present in 81% of samples and the average abundances ranged from 0 to 11100 MPN/100 ml. The annual average abundances of non-O1 *V. cholerae* were 4903 MPN/100 ml in all samples. Detectable *P. aeruginosa* was present in 88% of samples and its abundances ranged from 0 to 1670 cfu/100 ml with annual average densities of 206 cfu/100 ml. Throughout the year of study, the total incidence of FC and FS was 94%. The considerable variations of their densities were respectively recorded from a minimum of 0 cfu/100 ml to a maximum of 10200 cfu/100 ml for FC and 6700 cfu/100 ml for FS. Nevertheless, the annual mean abundances of FC and FS were respectively 1891 cfu/100 ml and 1246 cfu/100 ml. Among the studied physical parameters, the water temperature was important to define cold and hot periods. The average water temperature during the whole period of our study was 22 °C. Temperature values varied between 15 °C and 30 °C. Based on the average water temperature, two periods were defined: October to March (cold period: $T < 22$ °C) and April to September (hot period: $T \geq 22$ °C). This has served to scrutiny the temporal evolutions of non-O1 *V. cholerae*, FC, FS, and *P. aeruginosa* which generally appeared to be similar. Relatively, their highest densities were noted during the hot period, while their low levels were noted during the cold period. The average abundances of non-O1 *V. cholerae* were 7696 MPN/100 ml in the hot period and 2109 MPN/100 ml in the cold period. The hot period was characterized by average abundances of FC of 3083 cfu/100 ml and 699 cfu/100 ml in the cold period. FS registered 1821 cfu/100 ml in the warm season and 671 cfu/100 ml in the cool season. But, *P. aeruginosa* was distinguished by mean densities of 308 cfu/100 ml in the warm period and 104 cfu/100 ml in the cold period.

For non-O1 *V. cholerae* and FC, W9 was the most contaminated. This well is located near a municipal landfill and sewage effluent. The bacterial abundances were compared in the studied wells. The average densities of FC, FS, *P. aeruginosa*, and non-O1 *V. cholerae* were very significantly ($p < 0.05$, ANOVA test) higher in the whole wells compared to the control well (W11). On the other hand, indicators of fecal contamination showed significantly higher abundances than those of *P. aeruginosa* ($p < 0.05$, ANOVA test). *P. aeruginosa* is requiring more interest as an indicator in the assessment of swimming pool water quality, drinking water, recreational and wastewater [39, 40]. This opportunistic pathogen provides differentiation of the human origin feces most likely rather than animal origin feces in waters [41]. All of these results demonstrated that the studied well waters were heavily contaminated with non-O1 *V. cholerae*, indicators of fecal contamination, and *P. aeruginosa*. The prospected wells play a crucial role in the supply of rural and suburban populations

in the Marrakesh area to meet their needs for drinking water, domestic water supply, and recreation activities. This fact points out potential health effects on populations using them directly without any previous treatment.

The comparison of these bacterial abundances in the different sampling locations has shown that the most loaded wells were surrounded by several sources of pollution such as septic pit, manure, wastes, spreading wastewater, animal stools and detergents. Based on the results of our study, it is possible to conclude that groundwater can play an important role as a transmission vehicle of non-O1 *V. cholerae* and the other studied bacteria. Our findings are in agreement with those reported by Nogueira et al. [42] and Isaac-Marquez [17]. These authors investigated water quality at sources and points of consumption of urban and rural communities. According to them, the water distribution system, spring water and private wells samples had high coliforms positive and high percentages of non-O1 *V. cholerae*. Several reports have demonstrated that gastrointestinal and extraintestinal infections caused by non-O1 *V. cholerae* are linked with contaminated water and other activities in aquatic environments, and this bacterium could therefore pose a problem for public health [43, 44]. In this study, it appears that the contamination of the two prospected aquifers was not only due to a simple process of diffusion but also to the various sources of pollution around the wells. The wells situated close to many sources of pollution showed the greatest densities of the studied bacteria. These sources of contamination include faulty septic systems, landfill leachates, and infiltration of untreated sewage. Also, septic tanks seepage or runoffs of human and animal activities nearby the studied wells have an impact on their safety. All of these factors led to the contamination of the groundwater. The majority of the studied wells are situated 20 to 400 m from pollution sources. Furthermore, Tensift and Jbilet aquifers are found under the highly porous texture of the ground, allowing rapid infiltration of contaminants into groundwater. Next to that the extreme permeability subsurface formations generate hydraulic fracturing made up through cracks, root channels, and fissures in these sloping areas. The nature of these subsurface formations produces an accelerated flow of pollutants derived from the surface build-up of solid waste and sewage. The shallowness of the monitored wells is also involved in the contamination of Tensift and Jbilet groundwaters. In addition, these unprotected dug wells facilitate the introduction of polluting substances. These combined factors revealed the vulnerability of Tensift and Jbilet groundwater. Besides, the obtained results indicated that the distributions of FC, FS, *P. aeruginosa*, and non-O1 *V. cholerae* undergo spatio-temporal fluctuations. Their abundances increased in the hot season and were lower in the cold season. The effects of the temperature on the microbial growth rate, lag phase, and cell yield have been defined in previous studies [45]. Indeed, *Pseudomonas putida* presented a shorter growth phase lag of only 10 h at 17.5 °C but was longer within 3 days at 7.5 °C [45].

To evaluate the impact of some chemical factors on the evolutions of the studied bacterial groups, the evolutions of physicochemical parameters were followed. According to the survey, the water pH of these two studied aquifers remained neutral or slightly basic and varied from 6.8 to 8.1. Total mineralization of the studied water wells was expressed by electrical conductivity. Its values average showed that the prospected wells were fair to greatly mineralized. Moreover, the wells situated downstream are the most loaded. When conductivity values exceed 2000 μS/cm, water presents laxative effects for the consumers [46]. The water of the two-studied groundwater presented strong concentrations of major elements (Ca^{2+}, Na^{+}, Cl^{-}, SO_4^{2-} and K^{+}) with high concentrations of nitrogenous ions. They were particularly very hard, very salt, very chlorinated, and present high concentrations of sulphate. According to the international standard limits, the gauged concentrations of

nitrites, calcium, sodium, and chloride overtake these requirements [47]. Seasonal impacts are expressed by ammonium, nitrites, calcium, and organic matter. Their fluctuations were similar to those of the counted bacterial groups, but there was no temporal pattern for the other chemical parameters. These influences result from rainwater runoff and temperature variation that upload greatly calcium, ammonium, nitrites, and organic matter from soil to groundwater during the wet season compared to the dry season.

Based on the above results, the relationship between bacteriological and physicochemical parameters was determined using the Spearman correlation test. The studied wells are a dynamic system where bacteriological and chemical components might interact in a complex synergistic or antagonistic manner. Analysis of potential correlations between the different abundances of non-O1 *V. cholerae* and FC reflected that there is a stronger linkage among them in the studied wells. FC can testify to the existence of non-O1 *V. cholerae* in Marrakesh groundwater. However, no significant statistical correlation could be shown between the concentrations of non-O1 *V. cholerae* and *P. aeruginosa*. Several studies were conducted for the purpose of correlating the densities of fecal indicators with the presence of pathogens. Gallacher and Spino [48] emphasized that a correlation between the levels of total and fecal coliforms and the possibility of isolating pathogens would be valuable in setting reliable bacteriological standards, particularly for recreation and fishing uses. Hoi et al. [49] also demonstrated significant correlations between the occurrence of coliform bacteria and that of FS and also between coliform bacteria and *Vibrio vulnificus*. However, Mezrioui et al. [33] and Mezrioui and Oufdou [12] have shown that the spatial–temporal dynamic of non-O1 *V. cholerae* abundances was reverse to that of FC in other ecosystems such as sewage waters or water purified by the stabilization pond system.

The simulation of the sixteen well water points to a single ecosystem indicates a strong and positive relationship between calcium and FC and FS abundances. It was also indicated that a synergistic relationship exists between non-O1 *V. cholerae*, salinity, chlorides, calcium, sulphates, and nitrates ($p < 0.05$) especially after the mock of the six wells water like a single ecosystem. However, there is a significant negative correlation between calcium and *P. aeruginosa* abundances. These same correlations between nitrites and FC, FS, and *P. aeruginosa* abundances were recorded. Sjogren and Gibson [50] have revealed an important effect of acidic pH linked with the concentrations of calcium, magnesium, and other ions commonly present in hard-water, on the survival of enteric bacteria in the environment. These elements contributed to the adsorption of Fe^{3+} and Al^{3+} in the environment [51]. Ca^{2+} or Mg^{2+} are necessary for the physical integrity of many microbial components and intervene in some *Bacillus* species metabolism in the organic molecules transportation through the cell wall [52].

2.2 Pathogenesis of non-O1 *V. cholerae*

Infections caused by pathogenic *Vibrios* remain a severe threat to the public. Most of these infections result from the consumption of contaminated water or undercooked seafood products [53]. Non-O1 *V. cholerae* can also cause infections to range from self-limiting gastroenteritis to severe life-threatening septicemia and necrotizing fasciitis [53]. However, their infections are under-detected and under-reported because clinicians and microbiologist underestimate their abilities to induce diarrhea, and given that searching non-O1 *V. cholerae* is not subjected to routine testing [54]. Between 1 and 3.4% of cases of acute diarrhea are believed to be due to non-O1 *V. cholerae*, in developing and developed countries alike [55]. Up to now, there are few reports of the frequency of isolation of

non-O1 *V. cholerae* and there is an under-diagnosis of their infections, especially for those taken as milder cases, from the consumption of contaminated groundwater supplies in Morocco. The significant abundances of non-O1 *V. cholerae*, FC, FS, and *P. aeruginosa* recorded in this work confirm the presence of pathogenic microorganisms in groundwater consumed by the suburban and rural population in Marrakesh area. So that also informed the need to conduct the study about their virulence factors.

The enteropathogenicity of non-O1 *V. cholerae* is multifactorial [56]. The various putative virulence determinants identified in non-O1 *V. cholerae* include the production of cholera toxin CT [57, 58], enterotoxin LT or ST [59, 60], *V. cholerae* cytolysin (VCC) referred to a hemolysin and cytolysin with activity against a range of eukaryotic cells [61], shiga-like toxin [62] and cell-associated hemagglutinins [63]. It has been demonstrated that non-O1 *V. cholerae* adheres and invades the epithelial cells of gut mucosa and starts its multiplication [64]. It includes mild watery diarrhea of 1 or 2 days duration, a severe dehydrating disease resembling cholera, and dysentery [65]. This situation occurs only with the expression of certain virulence factors as previously cited [60–62, 64]. Alternatively, *V. cholerae* virulence factors encoded on a mobile genetic element, can spread through horizontal transfer. This underscores the importance of the environmental strains of non-O1 *V. choleare* as a reservoir of virulence genes that generate the dissemination of new variants [66].

In this work to characterize the virulence factors of the bacterial isolates recovered during our study, hemolysis and hemagglutination with human erythrocytes were realized. The potential virulence of non-O1 *V. cholerae* in supplying well waters in comparison with *P. aeruginosa*, FC, and FS. To our knowledge, this is the first report on the incidence of hemolytic and hemagglutination activities and antibiotic resistance of bacteria isolated from rural and suburban well waters from Morocco and particularly in Marrakesh groundwater. For hemolytic activity and hemagglutination assays 317 strains of non-O1 *V. cholerae*, 208 strains of *P. aeruginosa*, 320 strains of FC, and 338 strains of FS were collected from the prospected wells over the year. After bacterial strains, isolation was streaked onto Trypticase Soy Agar (TSA) for purification. The ability of all these isolates to adhere and destroy host cells was verified with human O erythrocytes. Quantification of hemolytic activity of bacterial cells was performed according to the procedure described by Rahim et al. [67] and Lamrani Alaoui et al. [34]. The hemagglutination test was realized according to the methodology described by Pal et al. [68] and Lamrani Alaoui et al. [34]. To statistically analyze the percentage of hemolytic and hemagglutination activities, the test of two proportions or frequencies was carried out as referred by Schwartz [69].

This test enables comparisons and identification of significant differences between two frequencies: f_1 noted on n_1 samples and f_2 noted on n_2 samples as shown below:

$$t = \frac{f1 - f2}{\sqrt{f(1-f)\left(\frac{1}{n1} + \frac{1}{n2}\right)}} \text{ With } \frac{n1f1 + n2f2}{n1 + n2} \quad (1)$$

If $|t| < 1.96$: the difference between f1 and f2 was not significant ($p > 0.05$).

If $|t| \geq 1.96$: the difference was significant ($p \leq 0.05$).

Non-O1 *V cholerae*, *P. aeruginosa*, FC, and FS strains have distinct hemolysin production. Complete and partial hemolytic activities of non-O1 *V. cholerae* isolates indicate major percentages of 71.29%. Hemolysin productions, among FS and FC strains have reached respectively considerable levels of 20.71% and 16.88%

compared to *P. aeruginosa* isolates (9.13%). Non-O1 *V. cholerae* expressed significantly the greatest β hemolytic activity of 33.12%, while only 3.44% of FC and 4.44% of FS strains were β hemolytic ($p \leq 0.05$, the test of two proportions). The lowest β hemolytic activities were recorded among *P. aeruginosa* strains (1.44%). Hemolysin of *V. cholerae* is suggested to be a virulence factor contributing to pathogenesis [70]. Guhathakurta et al. [71] Purified a bifunctional hemolysin-phospholipase C molecule from non-O1 *V. cholerae* (O139) showing enterotoxic activity, as shown by fluid accumulation in the ligated rabbit ileal loop and in the intestine of suckling mice [68]. In this work, obtained hemolytic activity percentages were comparable to those registered by Begum et al. [72]. They noted that 80% of non-O1 and non-O139 *V. cholerae* strains were hemolysin producers. Nevertheless, our findings were inferior in number to those given by Amaro et al. [73]. They demonstrated that hemolytic activity within environmental non-O1 *V. cholerae* strains achieved a percentage of 97%. Adhesion to the intestinal mucosa represents the first step in the infectivity of bacterial pathogens such as *V. cholerae* [74].

The extent of the results of hemagglutination activities of the adhesive bacterial strains isolated from Marrakesh groundwater was fluctuating from 63.09% for non-O1 *V. cholerae* to 65.09% for FS, 84.06% for FC, and 87.98% for *P. aeruginosa*. From a total of 317 strains of non-O1 *V. cholerae*, 18.93% were highly adhesive and 44.16% did not agglutinate completely to the erythrocytes. Besides, 69.06% of FC strains and 62.02% of *P. aeruginosa* possessed a complete agglutination ability, and respectively 15% and 25.96% of them agglutinated partially. Our results are consistent with prior research related to the distribution of hemagglutination in environmental non-Ol *V. cholerae* [73]. They noted that 78% of the tested strains presented agglutinating ability. Baffone et al. [75] study on the identification of virulence factors among *V. fluvialis*, *V. alginolyticus*, non-O1 *V. cholerae*, and *V. parahaemolyticus* showed that their adhesive capability was respectively expressed with varying percentages of 40% to 55–80%.

2.3 Antimicrobial susceptibility of non-O1 *V. cholerae*

Antibiotics and other antimicrobial agents have been used, since their discovery, for the treatment and management of bacterial infections in humans and animals [76]. Waterborne bacterial pathogens such as *Vibrio* spp., *Escherichia coli*, *Pseudomonas*, fecal coliforms and *enterococci*, and other enteric pathogens cause diseases around the world and still with their increasing antibiotic resistance the major drinking water health threats in developing countries [35–37]. Disappointingly, the rapid overuse of recommended antimicrobials drives mainly to the development of drug-resistant pathogens. So, antibiotic resistance is a global health threat that requires more expensive medication and can compromise therapeutic success leading to morbidity and mortality [77, 78]. On the other side, many people, especially in rural areas, rely on untreated groundwater for their water supplies. Consumption of contaminated water is one of the sources of *Vibrio* infections. Among the other virulence factors, resistance to antibiotics is more important. Several authors have noted that *V. cholerae* species are rapidly adapting to new drugs commonly used in medicine [79, 80], becoming a potential risk to public health. In addition, molecular analyses demonstrated that resistance to antibiotics and the other virulence factors are chromosomally mediated [81, 82]. Few studies have been done to determine the antibiotic resistance of isolates from groundwater. Non-O1 *V. cholerae* and *Pseudomonas* were resistant to ampicillin, chloramphenicol, and streptomycin have been isolated [35, 83]. According to our knowledge, no study has been developed on the occurrence of antibiotic resistance of non-O1 *V. cholerae* in groundwater supplies in Morocco and particularly in Marrakesh area. For this reason, the other

objective was focused on the study of the antibiotic resistance of non-O1 *V. cholerae* in untreated suburban and rural well water of Marrakesh region. A comparison was made between the dynamics and antibiotic resistance of non-O1 *V. cholerae* and those of fecal coliforms (FC).

In order to estimate the sanitary risk associated with antibiotic resistant bacteria, a total of 317 strains of non-O1 *V. cholerae*, 320 strains of FC, 338 strains of FS, and 204 strains of *P. aeruginosa* were collected from the prospected wells over the year and were tested for their antibiotic susceptibility. The strain antibiotic resistance study was carried out using the multipoint inoculation method reported by Oufdou et al. [84] and Lamrani Alaoui et al. [34]. The antibiotic was incorporated into molten Muller-Hinton agar in order to prepare the plates. After their inoculation, they were incubated at 37 °C for 24 h.

The control plate was elaborated without antibiotic and was inoculated in the same way. In comparison with the control plate and if no growth of the strain is observed in the medium containing the concentration of antibiotic tested the bacterium is considered resistant. The concentrations (given in μg ml^{-1}) of the antibiotics tested have been cited in the above studies. These antibiotics were chosen for two reasons: (i) they have been used in the treatment of human and/or livestock illnesses; and (ii) they have been used in previous surveys of antibiotic resistance in aquatic environments [12, 84–86]. Similarly, the test of two proportions or frequencies described by Schwartz [69] was used to compare percentages of antibiotic resistance between non-O1 *V. cholerae*, FC, FS, and *P. aeruginosa*. Obtained results showed that the overall resistance (resistance to at least one antibiotic) of non-O1 *V. cholerae* strains was 79%, while it was 100% for *P. aeruginosa*, FC, and FS strains. The mono-resistance (resistance to one antibiotic) of non-O1 *V. cholerae* was 10% while it was 5% for FC and FS strains. The multi-resistance of non-O1 *V. cholerae* strains remain at a degree (69%) significantly below those of FC and FS strains (95%) ($p < 0.05$, the test of two proportions), while all *P. aeruginosa* strains were multi-resistant. On the other hand, the multi-resistance of non-O1 *V. cholerae* and FC strains was significantly higher ($p < 0.05$, the test of two proportions) than that of their monoresistance. The susceptibility of non-O1 *V. cholerae* to all antibiotics tested is estimated at 21%, while none of the isolates *P. aeruginosa*, FC and FS was susceptible to all antibiotics tested. Our findings highlighted the most common antibiotics for which non-O1 *V. cholerae* strains resistance is recorded are in ascending order: trimethoprim (49%), cephalothin (60%), streptomycin (62%) and sulfamethoxazole (75%). The lowest proportions of resistance were toward erythromycin (18%), kanamycin and polymyxin B (12%), cephotaxim (8%), gentamycin (7%), and tetracycline (2%). It's important to perceive that all the 317 non-O1 *V. cholerae* isolates were susceptible to chloramphenicol, nalidixic acid, and novobiocin. Amaro et al. [73] have demonstrated that non-O1 *V. cholerae* environmental isolates were resistant to sulfanilamide (80%), to ampicillin (63%) and to amoxicillin (61%) and were susceptible to chloramphenicol, nalidixic acid, tetracycline, novobiocin and trimethoprim. Radu et al. [87] showed that all *V. cholerae* isolates were susceptible to chloramphenicol and exhibited high rates of resistance to cephalothin (90.9%), streptomycin (87.9%), and tetracycline (77.79%). Isolates of non-O1 *V. cholerae* from the aquatic environment in India were found to have multiple antibiotic resistance. Thirty-nine percent of *V. cholerae* isolates were resistant to two drugs [88]. Furthermore, several cases of antimicrobial resistance have been described in environmental as well as in clinical strains, involving cefotaxime, nalidixic acid, tetracyclines, cotrimoxazole, ciprofloxacin, and depending on location, certain multidrug resistant strains having been reported [55, 89]. Nevertheless, FC results are in perfect agreement with the findings of McKeon et al. [10] indicating that the overall resistance of the coliforms is around 87%. This one comprises respectively 14, 64, and 94% of *E. coli*, *Citrobacter freundii*

and *Enterobacter cloacae* isolates. The multi-resistance was registered by all bacterial strains isolated from groundwater and fluctuated as great as 100% for *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Klebsiella ozonae*. These authors have also demonstrated that 100% of the non-coliforms isolated from groundwater were multiple antibiotics resistant. El-Zanfaly et al. [90] observed a high multiple antibiotic resistance (63%) for gram-negative bacteria isolated from rural waters. Amundsen et al. [91] noted that 45% of total coliforms isolated from untreated well water were multidrug-resistant.

Antimicrobial resistance of FC strains was most widely manifested against sulfamethoxazole (91%), followed by cephalothin (88%) and ampicillin (84%). More than 30% of the FC isolates were resistant to kanamycin, gentamycin, streptomycin, trimethoprim, and tetracycline. However, the smaller susceptibility proportion was noted to chloramphenicol (13%) and nalidixic acid (28%). FS isolates were resistant to polymyxin (87%), sulfamethoxazole (86%), and nalidixic acid (85%), cephalothin (82%), and to streptomycin (74%), while they were less resistant to ampicillin (28%) and amoxicillin-clavulanic acid (34%). 28%, 34%, and 40% of FS isolates were resistant respectively to ampicillin, amoxicillin-clavulanic acid, and cephotaxim. The highest prevalence of resistance was observed among *P. aeruginosa* strains. More than 90% of these isolates were resistant to cephalothin (95%), cephotaxim (93%), polymyxin (92%), and cephamandole (90%). Also, 86% of *P. aeruginosa* isolates were resistant to tetracycline and nalidixic acid, 83% to ampicillin and kanamycin, 81% to streptomycin and 80% to trimethoprim, respectively. *P. aeruginosa* strains were generally resistant to the antibiotics tested, whereas they were less resistant only to imipenem (12%). The predominant resistance property observed was to β-lactam antibiotics, either alone or in combination with resistance to other antibiotics. Bell et al. [92] have also noted ampicillin and cephalothin resistance exhibited by most fecal coliform strains isolated from both rural and urban environments. McKeon et al. [10] analyzed antimicrobial susceptibility toward sixteen antibiotics of 265 coliform and non-coliform strains isolated from rural groundwater. They established that the overall resistance was approximately 70% and frequently indicated against novobiocin, cephalothin, and ampicillin. They have found that resistance toward tetracycline and nitrofurantoin was more than 30%.

The main multi-resistant patterns identified for non-O1 *V. cholerae* were to seven antibiotics with a percentage of around 24.09%. These strains were characterized with six profiles to seven antibiotics while the maximal multidrug resistance was to ten antibiotics with two profiles: "Gm, Str, Km, Tpm, Smx, Amp, Amx, Cfl, Cfm, Ery", and "Gm, Str, Km, Tpm, Smx, Tc, Amp, Amx, Cfl, Cfm". For the isolated FC, antimicrobial susceptibility toward five or more antibiotics was registered by 80% of them. The prevalent multi-resistant profile recorded for FC was to eight antibiotics (11.6%). The maximal multidrug resistance was to fourteen antibiotics with two patterns: "Amp, Amx, Amx-clav, Cfl, Cfm, Cft, Gm, Km, Na, PB, Smx, Str, Tc, Tpm" and "Amp, Amx, Amx-clav, Cfl, Cfm, Cft, Chl, Gm, Na, PB, Smx, Str, Tc, Tpm".

3. Conclusion

Groundwater is the primary drinking water source for a large suburban and rural population in Morocco. Hence, it is critical to ensure that groundwater resources are protected and of acceptable drinking quality. To the best of our knowledge, this study presents the most comprehensive monitoring of non-O1 *V. cholerae*, *P. aeruginosa*, and fecal indicator bacteria from Marrakech wells waters. Our findings

show that microbial presence within Marrakech groundwater supplies is a persistent issue, with a total occurrence of 81% for non-O1 *V. cholerae* and 94% for FC, FS, and *P. aeruginosa*. Another ecological challenge was elucidated since these bacteria with other physicochemical parameters underwent generally spatial and temporal fluctuations that structure bacterial variability. The temporal evolutions of non-O1 *V. cholerae* and FC appeared to be similar. Relatively, the highest densities were noted during the hot period, while the low levels were noted during the cold period. This heavy colonization of the prospected wells, with potentially pathogenic bacteria, implies that they are unsuitable for drinking and other domestic activities according to the international norms. Also, a highly significant relationship was observed between non-O1 *V. cholerae* and FC abundances in the studied wells. FC can be used to detect the presence of non-O1 *V. cholerae* in Marrakesh groundwater. Generally, no significant correlation was detected between these fecal indicator bacteria and *P. aeruginosa*. This opportunistic pathogenic bacterium is acquiring greater importance and may be useful in evaluating groundwater quality. The degrees of antibiotic resistance and particularly of multi-resistance of non-O1 *V. cholerae*, FC, FS, and *P. aeruginosa* strains are high. The multi-resistance of non-O1 *V. cholerae* strains (69%) was significantly lower than that of FC strains (94%). Non-O1 *V. cholerae*, FC, FS, and *P. aeruginosa* strains resistant to antibiotics occurred during the whole study period. The multiple antibiotic resistant bacteria once introduced into the studied groundwater supplies are capable of long-term survival in this ecosystem. In addition, non-O1 *V. cholerae* and the other studied bacteria isolated from Marrakesh groundwater are virulent since most of them are producers of hemolysins, hemagglutinins. These wells are used directly without any previous treatment for drinking water supply and domestic and recreation activities of these rural and suburban populations. This fact could be at the origin of the emergence of diarrheal diseases noted among them. It is well known that the incidence of multiple antibiotic resistant bacteria is a cause for concern because of possible colonization of the gastrointestinal tract and conjugal transfer of antibiotic resistance to the normal flora, serving to further amplify the number introduced into aquatic environments such as groundwater reservoirs. The ability of non-O1 *V. cholerae* and the other bacteria to survive in groundwater throughout the period of the study indicated a continuing trend of human and animal fecal contamination. The leachates from the reservoir of animal fertilizer which contaminate the groundwater might be a source of antibiotics in this water ecosystem. The manure runoff, leakage from septic tanks or broken sewage channels, led to the input of antibiotic resistant bacteria into groundwater. Given that the groundwater may play an important role in the spread of resistant and virulent bacteria, further study using molecular tools is warranted to properly evaluate the public health and ecological significance of these antibiotic and virulent bacteria in rural drinking water supplies. Non-O1 *V. cholerae* and the other bacteria could act as a reservoir of resistance and virulence genes in the groundwater environment.

Ultimately, the study highlights the need to remain highly suspicious of non-O1 *V. cholerae* infections related to consumption of contaminated wells water with known risk factors. Adaptation of special strategies should be taken to avoid poor maintenance, inappropriate well location and a historical lack of regulation that has led to many instances of groundwater contamination and associated public health issues. Consumption of these wells water requires an urgent reaction to apply adequate solutions. The protection of these wells waters, implementation and management of sanitation systems and sewerage network, the disinfection of groundwater should be adopted to cope with unforeseen situations and to decrease the water-related disease burden. As shown in reported results, this approach is still valuable in indicating potential avenues for future research. It's vital to link education and

social awareness with these measurements which play a major role in confronting and controlling groundwater pollution, water-related diseases, and subsequently in improving the human health of these suburban and rural populations.

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Author details

Hafsa Lamrani Alaoui[1*], Khalid Oufdou[2] and Nour-Eddine Mezrioui[2]

1 Laboratory of Microbiology, Faculty of Medicine and Pharmacy, Mohammed V University in Rabat, Rabat, Morocco

2 Laboratory of Microbial Biotechnologies, Agrosciences, and Environment, Faculty of Sciences, Semlalia, Cadi Ayyad University, Marrakesh, Morocco

*Address all correspondence to: h.alaoui@um5s.net.ma

References

[1] Reig, P., Maddocks, A., Gassert, F. (2013). World's 36 most Water Stressed Countries Data. World Resources Institute, Available online: http://www.wri.org/blog/2013/12/world%E2%80%99s-36-most-waterstressed-countries (accessed on 12 January 2021).

[2] Rijsberman, F.R. (2006). Water scarcity: Fact or fiction? Agricultural Water Management, 80: 5-22.

[3] Maddocks, A., Young, R.S., Reig, P. (2015). Ranking the World's most Water-Stressed Countries in 2040. World Resources Institute: Washington, DC, USA, Available online:http://www.wri.org/blog/2015/08/ranking-world%E2%80%99s-most-water-stressed-countries-2040 (accessed on 12 January 2021).

[4] Vineis, P., Chan, Q., Khan, A. (2011). Climate change impacts on water salinity and health. Journal of Epidemiology and Global Health, 1: 5-10.

[5] Abedin, M.A., Collins, A.E., Habiba, U., Shaw, R. (2019). Climate change, water scarcity, and health adaptation in southwestern coastal Bangladesh. International Journal of Disaster Risk Science, 10: 28-42.

[6] World Health Organization. Drinking Water. Fact Sheet. (2016). Available online: http://www.who.int/ mediacentre/factsheets/fs391/en/ (accessed on 12 January 2021).

[7] Ashbolt, N.J. 2004. Microbial contamination of drinking water and disease outcomes in developing regions. Toxicology, 198: 229-238.

[8] Bouhoum K., Habbari Kh., Jana M. (1997). Etude épidémiologique des helminthiases intestinales chez l'enfant de la zone d'épandage des eaux usées de la ville de Marrakech. In "Conception, naissance et petite enfance au Maghreb". Cahier de l'IREMAN, Institut de recherche et d'étude sur le monde arabe et musulman, CNRS-Univ. Aix-Marseille, 9/10: 275-291.

[9] Ait Melloul, A., Hassani, L. (1999). Antibiotic resistance of Salmonella strains isolated from children living in the wastewater spreading area of Marrakesh city (Morocco). World Journal of Microbiology and Biotechnology, 15: 91-96.

[10] McKeon, M.D., Calabrese, J.P., Bissonnette G.K. (1995). Antibiotic resistant gram-negative bacteria in rural groundwater supplies. Water Research, 29: 1902-1908.

[11] Arvanitidou M., Katsouyan nopoulos V., Tsakris A. (2001). Antibiotic resistance patterns of enterococci isolated from coastal bathing waters. Journal of Medical Microbiology, 50: 1001-1005.

[12] Mezrioui, N., Oufdou K. (1996). Abundance and antibiotic resistance of non-O1 *Vibrio cholerae* strains in domestic wastewater before and after treatment in stabilization ponds in an arid region (Marrakesh, Morocco). FEMS Microbiology Ecology, 21: 277-284.

[13] Wellington, E. M., Boxall, A. B., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., Johnson-Rollings, A. S., Jones, D. L., Lee, N. M., Otten, W., Thomas, C. M., A. P. Williams. (2013). The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. The Lancet Infectious Diseases, 13(2):155-65.

[14] Grimes, D. J., Johnson, C. N., Dillon, K. S., Flowers, A. R., Noriea III, N. F., Berutti, T. (2009). What genomic sequence information has revealed

about *Vibrio* ecology in the ocean-a review. Microbial Ecology, 58: 447-460. doi:10.1007/s00248-009-9578-9.

[15] Johnson, C.N. (2013). Fitness factors in *Vibrio*s: a mini-review. Microbial Ecology, 65: 826-851. doi:10.1007/s00248-012-0168.

[16] Colwell, R.R. (2004). Infectious disease and environment: cholera as a paradigm for waterborne disease. International Microbiology, 7: 285-289.

[17] Isaac-Marquez, A. P., Lezama-Davila, C. M., Eslava-Campos, C., Navarro-Ocana, A., Cravioto-quintana, A. (1998). Serotype of *Vibrio cholerae* non-O1 isolated from water supplies for human consumption in Campeche, Mexico and their antibiotic resistance susceptibility pattern. Memorias do Instituto Oswaldo Cruz, Rio de Janeiro, 93: 17-21.

[18] Haley, B.J. , Choi, S. Y., Grim, C. J., Onifade, T. J., Cinar, H. N., Tall, B. D., Taviani, E., Hasan N. A., Abdullah, AS. H., Carter, L., Sahu, S. N., Kothary, M. H., Chen, A., Baker, R., Hutchinson, R., Blackmore, C., Cebula, T. A., Huq, A., Colwell, R. R. (2014) Genomic and phenotypic characterization of *Vibrio cholerae* non-O1 isolates from a US Gulf Coast cholera outbreak. PLoS ONE 9: (4) e86264

[19] Deshayes, S., Daurel, C., Cattoir, V., Parienti, J. J., Quilici, M-L., de La Blanchardière A.. 2015. Non-O1, non-O139 *Vibrio cholerae* bacteraemia: case report and literature review. Springer Plus, 4(575): 1-9. DOI 10.1186/s40064-015-1346-3.

[20] Kueh, C.S.W., Kutarski, P., Brunton, M. (1992). Contaminated marine wounds: the risk of acquiring acute bacterial infection from marine recreational beaches. Journal of Applied Bacteriology, 73: 412-420.

[21] Petsaris, O., Nousbaum, J.B., Quilici, M.L., Le Coadou, G., Payan, C., Abalain, M.L. (2010). Non-O1, non-O139 *Vibrio cholerae* bacteraemia in a cirrhotic patient. Journal of Medical Microbiology, 59:1260-1262.

[22] Kaki, R., El-Hossary, D., Jiman-Fatani, A., Al-Ghamdi, R. (2017). Non-O1/non-O139 *Vibrio cholerae* septicaemia in a Saudi man: a case report. JMM Case Reports, 4: 1-6 DOI 10.1099/jmmcr.0.005077

[23] Xie, H., Wu, Y., Liu, C., Guo, J., Ma, J., Li, X., Sun X. (2020). Oral infection caused by non-O1/non-O139 *Vibrio cholerae* in a patient with esophageal cancer undergoing esophagectomy and chemoradiotherapy: A case report. Infection and Drug Resistance, 13: 3923-3927.

[24] Banerjee, K. K., Ghosh, A. N., Dutta-Roy, K., Pal, S. C., Ghose, A. C. (1990). Purification and characterization of a novel hemagglutinin from *Vibrio cholerae*. Infection and Immunity, 58: 3698-3705.

[25] Dalsgaard, A., Albert, M.J., Taylor, D.N., Shimada, T., Meza, R., Serichantalergs, O. Echeverria, P. (1995). Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhoea in Lima, Peru. Journal of Clinical Microbiology, 33: 2715-2722.

[26] Sharma, C., Thungapathra, M., Ghosh, A., Mukhopadhyay, A.K., Basu, A., Mitra, R., Basu, I., Bhattacharya, S.K., et al. (1998). Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. Journal of Clinical Microbiology, 36: 756-763.

[27] Craun, G. F., Berger, P. S., Calderon, R. L. (1997). Coliform bacteria and water borne disease outbreaks. *Journal* (*American Water Works Association*), 89: 96-104.

[28] Dumontet, S., Krovacek, K., Svenson, S. B., Pasquale, V., Baloda, S. B, Figliuolod, G. (2000). Prevalence and diversity of *Aeromonas* and *Vibrio* spp. in coastal waters of Southern Italy. Comparative Immunology, Microbiology and Infectious Diseases, 23: 53-72.

[29] Lamrani Alaoui, H., Oufdou, K., Mezrioui, N. (2008). Environmental pollutions impacts on the bacteriological and physicochemical quality of suburban and rural groundwater supplies in Marrakesh area (Morocco). Environmental Monitoring and assessment, 145: 195-207.

[30] Boulanouar M., Yacoubi-khebiza M., Messouli M., Coineau N. (1995). Un nouveau Microcharbon (Isopoda, Janiroidea) du Maroc-Origine et biogéographie historique. Contribution to Zoology, 65: 53-64.

[31] Hakkou R., Wahbi M., Bachnou A., Elamari K., Hanich L., Hibti M. (2001). Impact de la décharge publique de Marrakech (Maroc) sur les ressources en eau. Bulletin of Engineering Geology and Environment, 60: 325-336.

[32] El Omari L. (1975). Note géothermique concernant le périmètre urbain de Marrakech et ses environs. Rap. SRG/ MAR / N° 36 Ministère du Commerce de l'industrie des Mines et de la marchandise.

[33] Mezrioui N., Oufdou K., Baleux B. (1995). Dynamics of non-O1 *Vibrio cholerae* and fecal coliforms in experimental stabilization ponds in the arid region of Marrakesh, Morocco, and the effect of pH, temperature and sunlight on their experimental survival. Canadian Journal of Microbiology, 41: 489-498.

[34] Lamrani Alaoui, H., Oufdou, K., Mezrioui, N. (2010). Determination of several potential virulence factors in non-o1 *Vibrio cholerae*, *Pseudomonas aeruginosa*, fecal coliforms and streptococci isolated from Marrakesh groundwater. Water Science and Technology, 61 (7): 1895-1905.

[35] Gangle, J.B. (2005). Sources and occurrence of antibiotic resistance in the environment. Thesis submitted to the Faculty of the Graduate School of the University of Maryland, College Park in partial fulfillment of the requirements for the degree of Master of Science, 125p

[36] Hynds, P. D., Thomas, M. K., Pintar K.. (2014). Contamination of Groundwater Systems in the US and Canada by Enteric Pathogens, 1990-2013: A Review and Pooled-Analysis. PLoS ONE, 9 (5): e93301.

[37] Pons, W., Young, I., Truong, J., Jones-Bitton A., McEwen, S., Pintar, K., Papadopoulos A. (2015). A systematic review of waterborne disease outbreaks associated with small non-community drinking water systems in Canada and the United States. PLoS ONE, 10(10): e0141646.

[38] Costerton, J.W., Stewart, P.S., Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. Science, 284: 1318-22.

[39] Bahlaoui , M.A., Baleux, B., Troussellier, M. (1997). Dynamics of pollution-indicator and pathogenic bacteria in high-rate oxidation wastewater treatment ponds. Water Research, 31: 630-638.

[40] De Victorica, J., Galvan, M. (2001). *Pseudomonas aeruginosa* as an indicator of health risk in water for human consumption. Water Science Technology, 43, 49-52.

[41] Olivieri, V. P. (1982). Bacterial indicators of pollution. In: Pipes W.O. (ed.) Bacterial indicators of pollution. CRC Press, Boca Raton, FL, p. 21-41.

[42] Nogueira, G., Nakamura, C. V., Tognim, M. C. B., Abreu Filho, B. A., Dias Filho, B. P. (2003). Microbiological quality of drinking water of urban and rural communities. Review Saùde Pùblica, 37: 232-236.

[43] WHO (World Health Organization). (1993). Epidemic diarrhea due to *Vibrio cholerae* non-O1. Weekly Epidemiological Record, 68: 141-142.

[44] Chakraborty, S., Nair, G. B., Shinoda, S. (1997). Pathogenic *Vibrio*s in the natural aquatic environment. Reviews on Environmental Health, 12: 63-80.

[45] Fransolet, G., Delavalentina, D. J., Masschelein, W. J. (1985). Influence of temperature on bacterial development in water. Ozone Science, 7: 205-227.

[46] WHO (World Health Organization) (1986). Directives de la qualité pour l'eau de boisson. Vol. 2. Critères d'hygiène et documentation à l'appui. Genève: OMS, 341 p.

[47] Diop, E. S., Tandia, A. A. (1997). Qualité de l'eau de la nappe phréatique à Yeumbeul, Sénégal. Etude sur le terrain, CSI info N° 3 UNESCO, Paris, 27 pp.

[48] Gallacher, T., Spino, D. (1968). The significance of numbers of coliform bacteria as an indicator of enteric pathogens. Water Research, 2: 169-175.

[49] Hoi, L., Larsen, J., Dalsgaard, I., Dalsgaard, A. (1998). Occurrence of *Vibrio vulnificus* biotypes in Dannish marine environments. Applied and Environmental Microbiology, 64: 7-13.

[50] Sjogren, R. E., Gibson, M. J. (1981). Bacterial survival in a dilute environment. Applied and Environmental Microbiology, 41: 1331-1336.

[51] Dommergues, Y., Mangenot, F. (1970). In: Masson et Cie, editor. Soil microbial ecology. Paris, 796 pp.

[52] Pelmont, J. (1993). Bacterium and environment. Physiological adaptation. Presse universitaire de Grenoble ed., Grenoble, 899 p.

[53] Centers for Disease Control and Prevention (CDC). (2014). "National enteric disease surveillance: covis annual summary," Summary of Human *Vibrio* Cases Reported to CDC, Centers for Disease Control and Prevention, Atlanta, GA.

[54] Pierce, A. B., Broughton, S. J., Johnson, P.D., Grayson, M. L. (2000) *Vibrio cholerae* in Victoria. Medical Journal of Australia, 172:44-46

[55] Luo, Y., Ye, J., Jin, D., Ding, G., Zhang, Z., Mei, L., Octavia, S., Lan, R. (2013). Molecular analysis of non-O1/ non-O139 *Vibrio cholerae* isolated from hospitalized patients in China. BMC Microbiology, 13:52.

[56] Ramamurthy, T., Bag, P. K., Pal, A., Bhattacharya, S. K., Bhattacharya, M. K., Shimada, T., Takeda T., Karasawa, T., Kurazono H., Takeda Y., Nair G. B. (1993). Virulence patterns of *Vibrio cholerae* non-O1 strains isolated from hospitalized patients with acute diarrhoea in Calcutta, India. Journal of Medical Microbiology, 39:310-317.

[57] Spira, W. M., Fedorka-Cray, P. J. (1983). Production of cholera toxin-like toxin by *Vibrio mimicus* and non-O1 *Vibrio cholerae*. Infection and Immunity, 42: 501-509.

[58] Yamamoto, K., Takeda, Y., Miwatani, T. Craig, J. P. (1983). Purification and some properties of a non-O1 *Vibrio cholerae* enterotoxin that is identical to cholera enterotoxin. Infection and Immunity, 39: 1128-1135.

[59] Arita, M., Takeda, T., Honda, T. Miwatani, T. (1986). Purification and

characterization of *Vibrio cholerae* non-Ol heat-stable enterotoxin. Infection and Immunity, 52: 45-49.

[60] Ichinose, Y., Yamamoto, K., Nakasone, N. (1987). Enterotoxicity of El Tor-like hemolysin of non-O1 *Vibrio cholerae*. Infection and Immunity, 55: 1090-1093.

[61] Ruenchit, P., Reamtong, O., Siripanichgon, K., Chaicumpa, W., Diraphat, P. (2018). New facet of non-O1/non-O139 *Vibrio cholerae* hemolysin A: a competitive factor in the ecological niche. FEMS Microbiology Ecology, 94: (1).

[62] O'Brien, A. D., Chen, M. E., Holmes, R. K., Kaper, J., Levine, M. M. (1984). Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemlovticus* produce a *Shigella dysenteriae* 1 (Shiga)-like cytotoxin. The Lancet, 323(8368): 77-78.

[63] Yamamoto, T., T. Yokota. (1988). *Vibrio cholerae* non-O1: production of cell-associated hemagglutinins and *in vitro* adherence to mucus coat and epithelial surfaces of the villi and lymphoid follicles of human small intestines treated with formalin. Journal of Clinical Microbiology, 26:2018-2024.

[64] Nishibuchi, M., Seidler, R. J., Rollins, D. M., Joseph, S. W. (1983). *Vibrio* factors cause rapid fluid accumulation in suckling mice. Infection and Immunity. 40: 1083-1091.

[65] Spira, W. M., Daniel R. R. (1979). Biotype cluster on the basis of virulence characteristics in non-O group 1 *Vibrio cholerae*, p. 440-457. In N. Pierce and K. Takeya (ed.), Symposium on cholera: Bethesda 1979. Proceedings of the 15th Joint Conference U.S.-Japan Cooperative Medical Science Program, Cholera Panel. NIH publication no. 80-2003. National Institutes of Health, Bethesda, Md.

[66] Chakraborty, S., Mukhopadhyay, A. K., Bhadra R. K., Ghosh A. N., Mitra R., Shimada, T., Yamasaki, S., S. M. Faruque, Y. Takeda, Colwell R., Nair G. B. (2000). Virulence Genes in Environmental Strains of *Vibrio cholerae*. Applied and Environmental Microbiology, 66(9): 4022-4028.

[67] Rahim, Z., Sultan Aziz, K. M. (1994). Enterotoxigenicity, hemolytic activity and antibiotic resistance of *Aeromonas* spp. Isolated from freshwater prawn marketed in Dhaka, Bangladesh. Microbiology and Immunology, 38: 773-778.

[68] Pal, S., Datta, A., Nair, G. B., Guhathakurta, B. (1998). Use of monoclonal antibodies to identify phospholipase C as the enterotoxic factor of the bifunctional hemolysin phospholipase C molecule of *Vibrio cholerae* O139. Infection and Immunity. 66, 3974-3977.

[69] Schwartz, D. (1963). Méthodes statistiques à l'usage des Médecins et des Biologistes (Statistical methods used for the medicine and the biology), 3rd edition, Paris, Medecine Flammarion Sciences ISBN 2-257-10326-2.

[70] Nagamune, K., Yamamoto, K., Honda, T. (1995). Cloning and sequencing of a novel hemolysin gene of *Vibrio cholerae*. FEMS Microbiology Letters, 128: 265-269.

[71] Guhathakurta, B., Sasmal, D., Pal, S., Chakraborty, S., Nair, G. B. Datta, A. (1999). Comparative analysis of cytotoxin, hemolysin, hemagglutinin and exocellular enzymes among clinical and environmental isolates of *Vibrio cholerae* O139 and non-O1, non-O139. FEMS Microbiology Letters, 179: 401-407.

[72] Begum, K., Ahsan, C. R., Ansaruzzaman, M., Dutta, D. K., Ahmad, Q. S. Talukder, K. A. (2006). Toxin(s), other than cholera toxin,

produced by environmental non O1 non O139 *Vibrio cholerae*. Cellular and Molecular Immunology. 3: 115-121.

[73] Amaro, C., Toranzo, A. E., Gonzalez, E. A., Blanco, J., Pujalte, M. J., Aznar, R. & Garay, E. (1990). Surface and virulence properties of environmental *Vibrio cholerae* non-O1 from Albufera Lake (Valencia, Spain). Applied and Environmental Microbiology, 56: 1140-1147.

[74] Booth, B. A., Finkelstein, R. A. (1986). Presence of hemagglutinin/protease and other potential virulence factors in O1 and non-O1 *Vibrio cholerae*. Journal of the Infectious Diseases, 154: 183-186.

[75] Baffone, W., Citterio, B., Vittoria, E., Casaroli, A., Pianetti, A., Campana, R., Bruscolini, F. 2001 Determination of several potential virulence factors in *Vibrio* spp. isolated from seawater. Food Microbiology, 18: 479-488.

[76] Saga T., Yamaguchi K., "History of antimicrobial agents and resistant bacteria," Japan Medical Association Journal, vol. 52, pp. 103-108, 2009.

[77] Carlet, J. P., Collignon, D., Goldmann D., Goossens, H., Gyssens I. C., Harbarth, S., Jarlier, V., Levy, S. B., N'Doye B., Pittet, D., Richtmann, R., Seto, W. H., Van der Meer J. WM., Voss, A. (2011). "Society's failure to protect a precious resource: antibiotics" Lancet, 378(9788): 369-371,

[78] Finley, R. L., Collignon, P., Larsson, D. G. J. McEwen, S. A., Li, X-Z., Gaze, W. H., Reid-Smith, R., Timinouni, M., Graham, D. W., Topp, E. (2013). "The scourge of antibiotic resistance: the important role of the environment". Clinical Infectious Diseases, 57(5): 704-710.

[79] Garg, P., Chakraborty, S., Basu, I., Datta, S., Rajendran, K., Bhattacharya, T., Yamasaki, S., Bhattacharya, S. K., Takeda, Y., Nair, G. B., Ramamurthy, T. (2000). Expanding multiple antibiotic resistance among clinical strains of *Vibrio cholerae* isolated from 1992-7 in Calcutta. India. Epidemiology and Infection, 124: 393-399.

[80] Thiagarajah, J. R., Verkman, A. S. (2005). New drug targets for cholera therapy. Trends in Pharmacological Sciences, 26: 172-176.

[81] Yamamoto, T., Nair, G. B., Takeda, Y. (1995). Emergence of tetracycline resistance due to a multiple drug resistance plasmid in *Vibrio cholerae* O139. FEMS Immunology and Medical Microbiology, 11: 131-136.

[82] Trucksis, M., Michalski, J., Deng, Y. K., Kaper, J. B. (1998). The *Vibrio cholerae* genome contains two unique circular chromosomes. Proceeding of the National Academy of sciences of the United States of America. 95: 14464-14469.

[83] Louis, V.R., Russek-Cohen, E., Choopun, N., Rivera, I.N., Gangle, B., Jiang, S.C., Rubin, A., Patz, J.A., Huq, A., Colwell R. R. (2003). Predictability of *Vibrio cholerae* in Chesapeake Bay. Applied Environmental Microbiology, 69: 2773-2785.

[84] Oufdou, K., Mezrioui, N., Ait Melloul, A., Barakate, M. Ait alla, A. (1999). Effects of sunlight and *Synechocystis* sp. (picocyanobacterium) on the incidence of antibiotic resistance in waste water enteric bacteria. World Journal of Microbiology and Biotechnology. 15: 553-559.

[85] Kelch, W. J., Lee, J. S. (1978). Antibiotic resistance patterns of gram-negative bacteria isolated from environmental sources. Applied Environmental Microbiology, 36: 450-456.

[86] Hassani, L., Imziln, B., Boussaid, A., Gauthier, M. J. (1992).

Antibiotic-resistant *Escherichia coli* from wastewater before and after treatment in stabilization ponds in the arid region of Marrakesh, Morocco. Letters in Applied Microbiology, 15: 228-231.

[87] Radu, S., Vincent, M., Apum, K., Abdul Rahim, R., Benjamin, P. G., Yuherman Rusul, G. (2002). Molecular characterization of *Vibrio cholerae* O1 outbreak strains in Miri, Srawak (Malaysia). Acta Tropica, 83: 169-176.

[88] Pathak, S. P., Bhattacherjee J. W., Ray P. K. (1993). Seasonal variation in survival and antibiotic resistance among various bacterial populations in a tropical river. Journal of General and Applied Microbiology, 39: 47-56.

[89] Lu, B., Zhou, H., Li, D., Li, F., Zhu, F., Cui, Y., Huang, L., Wang, D. (2014). The first case of bacteraemia due to non-O1/non-O139 *Vibrio cholerae* from type 2 diabetes mellitus in mainland China. International Journal of Infectious Diseases, 25:116-118

[90] El-Zanfaly, H. T., Hosny, I., Fayez, M., Shaban, A. M. (1988). Incidence of antibiotic resistant bacteria in undergroundwater. Environment International, 14: 391-394.

[91] Amundsen, D., Lindholm, C., Goyal, S. M., Robinson, R. A. (1988). Microbial pollution of well water in south-eastern Minnesota. Journal of Environmental Science and Health, 23: 453-468.

[92] Bell, J. B., Elliott, G. E., Smith, D. W. (1983). Influence of sewage treatment and urbanization on selection of multiple resistance in faecal coliform populations. Applied Environmental Microbiology, 46: 227-232.